Ophthalmic Examinations: No treatment-related ocular effects, other than pale eyes, due to excessive hemorrhagic effects were reported.

Organ Weights: No effects on organ weights were noted.

Gross Pathology: Treatment-related gross pathological findings occurred at the injection site and included a dose-dependent increase in the incidence and severity of punctiform, patch like and/or firmly encapsulated hematomas in all treated groups compared to control.

Histopathology: Histological correlates of localized encapsulated hemorrhages, fresh diffuse patch like hemorrhages (often considerable in extent) and chronic inflammation were seen at all dose levels, with a dose-dependent increased incidence and severity in the treated groups. Histological findings of reductions in hemosiderin pigment of the spleen (high dose males and females and increased erythropoiesis in the spleen (high dose females), and bone marrow (high dose males and females) were also observed at the end of the study. Animals which underwent the 29 day recovery period exhibited fibrosis of the subcutis frequently with old and circumscribed hemorrhages in the process of resorption and with foreign body reactions and macrophages containing hemosiderin.

Plasma Levels of the Drug: Analysis of plasma concentrations of r-Hirudin showed that maximal plasma concentrations were attained at approximately 30 min following dosing in both males and females. Plasma concentrations in males and female at the 30 min time point were: 245 and 260 ng/ml at the 0.4 mg/kg dose; 1010 and 1107 ng/ml at the 2 mg/kg dose; and 7819 and 7214 ng/ml at the 10 mg/kg dose. Generally observed increases were linear and dose-dependent.

In conclusion, r-Hirudin administered subcutaneously, at doses of 0.4, 2.0, and 10 mg/kg was lethal at the 10 mg/kg high dose. Toxicological effects were limited to a dose-dependent increase in incidence and severity of subcutaneous hemorrhagic effects at the site of injection. Excessive hemorrhagic effects at the 10 mg/kg dose also resulted in an apparent compensatory increased erythropoiesis in the bone marrow and spleen (individual animals), with a reduction in hemosiderin pigment in the spleen noted in high dose females. No other target organs of toxicity were identified. Due to the local injection site lesions following subcutaneous injection of r-hirudin, at all doses tested, a no effect subcutaneous dose was not established. The maximum tolerated dose appeared to be 0.4 mg/kg.

MONKEY:

Intravenous Subacute (Bolus + 72 hr Infusion) Toxicity Study in Monkeys. (Study No. 11589 TSP)

Study Started: April 12, 1994

Study Completed: February, 13, 1995

GLP Requirements: A statement of compliance with the GLP regulations and quality assurance unit was included.

Animals: Wild caught cynomolgus monkeys; Age not specified; Males 2.9 ± 0.22 kg and Females 2.5 ± 0.44 kg (mean ± standard deviation).

Drug Batch No.: 616114011

Methods: Two groups of monkeys (3/sex/group) were administered bolus injections of r-hirudin (dissolved in isotonic saline) at doses of 0.2 and 0.4 mg/kg in a volume of 5 ml/kg, followed by 72 hour i.v. infusions with r-hirudin at doses of 0, (vehicle 0.9% isotonic saline) 0.1 and 0.15 mg/kg/hr at an infusion rate of 1 ml/kg/hr (i.e. total daily infusion doses of 2.6 and 4.0 mg/kg). A separate group of control animals (3/sex) received equal volumes of vehicle (isotonic saline) alone. The basis of dose selection was not indicated. Following dosing, monkeys underwent an 11 day observation period in which they were examined daily for mortality and clinical signs of toxicity. Body weights were determined at predose, day 1 of dosing, and twice per week until the end of the observation period and food consumption was estimated on a daily basis. Electrocardiographic examinations and blood pressure determinations were conducted at pretreatment and on days 4 and 14, 2-4 hours after the end of the infusion period under light anesthesia (20 mg/kg ketamine HCL, i.m. injection). Hematological and blood chemistry were determined in samples collected at pretreatment, at the end of the treatment period (Day 4) and again at the end of the observation period (Day 14) and urinalysis was performed on samples obtained at the end of the observation period (Day 14). Animals which died or were sacrificed at the end of the observation period underwent complete gross examinations with determination of organ weights. Microscopic examinations were also conducted on all tissues showing macroscopic lesions and all other tissues except eye with optic nerve, femoral bone with articulation, prostate, skeletal muscle, skin, spinal cord, tongue, trachea and vagina.

Results:

Observed Effects: No drug-related clinical signs of toxicity were observed.

Mortality: One of three control female monkeys was sacrificed on day 8 due to complications resulting from the surgical procedure. Otherwise no drug-related deaths were observed.

Body Weight/Food Consumption: At the end of the treatment period control male and female rats weighed 2.9 ± 2.6 g and 3.0 ± 0.69 kg, respectively. These values were not significantly different from groups treated with r-Hirudin. Similarly, r-hirudin had no adverse effects on food consumption.

Hematology/Bone Marrow: A treatment-related increase in APTT (control values of was observed on Day 4 in both sexes of both treatment groups. Other hematological effects noted in both treated and control animals on Day 4 included: moderate reductions in erythrocytes ; reduced hemoglobin concentrations ; reduced packed cell volumes ; increased white blood cells , increased leukocyte counts in males and in females) and increased fibrinogen levels (predose values of . These latter effects occurred at comparable incidence and severity in both the treated and control groups and thus, were not considered drug-related.

Blood Chemistry: Treatment-related changes in clinical chemistry included: 1) a transient (Day 4 only) reduction in Sodium levels (5%, relative to control values of 146.9 mmol/l) in the 0.15 mg/kg/hr high dose males; 2) moderate increases in triglyceride levels on Day-4 in males at the 0.1 mg/kg/hr dose (86%) and in males at the 0.15 mg/kg dose (143%), relative to mean control values of 1.1 ± 0.281 mmol/l and 3) increased total bilirubin levels (63% to 3.3 fold greater than predose values which ranged from 4 to 8 µmol/l) in 2 males each at the 0.10 and 0.15 mg/kg/hr doses.

Urinalysis: Urine samples taken at the end of the observation period showed no drug-related effects.

Physical Examinations/Ophthalmic and ECG Examinations: No treatment-related effects on ECG (lead I, II, and III), heart rates, or conduction times were noted and no effects on blood pressure were observed. Ophthalmic examinations were not indicated.

Organ Weights: No treatment related effects on organ weights were evident.

Gross Pathology: Monkeys in all groups showed gross pathological findings at the infusion site which included a thickening and/or induration of the adjacent or subcutaneous tissue. The incidence of lesions at the infusion site was comparable in control and treated groups and were considered to be related to mechanical injury induced by the infusion technique. One of 3 males at the 0.1 mg/kg/hr dose also had a gall bladder with blackish contents, adhesion of the all left lobes of the lung and reddish/purplish nodules in the lumen of the infusion site. Enlarged inguinal lymph nodes were observed in males of all groups and in 1 of 3 females at the 0.1 mg/kg/hr, while enlarged iliac lymph nodes were seen in all drug-treated males and in 1 of 3 females, each at the 0.10 and 0.15 mg/kg/hr dose levels compared to only 1 of 3 control males. In addition, a blackish colors of the inguinal lymph nodes (1 of 3 low dose males and 2 of 3 high dose males) and of the iliac lymph nodes (1 of 3 high dose males) were also noted. Finally, the kidneys of three females, one each in the control (sacrificed on Day 8), 0.10, and 0.15 mg/kg/hr dose groups showed reddish/purplish and/or grayish/whitish areas. Other gross findings occurred at equal incidence in control and treated groups and were not considered treatment-related.

Histopathology: Histological correlates to gross injection site lesions included: intimal fibroplasia of the infused vein, minimal to severe organized thrombus, minimal to moderate phlebitis and minimal to marked fibroplasia of the perivenous, periarterial and/or adjacent tissues. The said findings were seen in animals of all groups, with no evidence of a dose-relationship. Gross renal lesions observed in the 2 females (one each at the 0.1 and 0.15 mg/kg/hr doses) had histological correlates which included: glomerulonephropathy together with hemorrhage (for the 0.10 mg/kg female) and dilated tubules, sometimes flattened together with acidophilic casts, peritubular fibrosis and organized thrombus of the renal artery (for the 0.15 mg/kg/hr female). However, two control females (one sacrificed on day 8) also had organized thrombus. The control female killed on Day 8 also had glomerulonephropathy and renal infarction (due to vascular disturbances subsequent to erroneous catheterization of the renal artery). Thus, the observed renal histopathological effects in females were considered equivocal.

Plasma Levels of the Drug: Measurement of plasma drug levels was not indicated.

In conclusion, administration of r-hirudin to Cynomolgus monkeys by bolus injection (0.2 or 0.4 mg/kg) immediately followed by a 72-hour infusion at 0.10 or 0.15 mg/kg/hr, respectively, was well tolerated at both doses tested (i.e. maximum total daily doses of 2.6 and 4.0 mg/kg). At the 4.0 mg/kg dose a reversible decrease in sodium levels was observed, while both doses resulted in prolonged plasma APTT. No target organs of toxicity were identified. With the exception of the reductions in Sodium levels, the 4.0 mg/kg dose could be considered the no effect dose for the study.

15-Day I.V. Toxicity Study of r-Hirudin Followed by a 4-Day Recovery Period (Study No. 13326 TSP).

Testing Laboratory: Behringwerke AG
D-35001 Marburg-Lahn, Germany

Compliance with Good Laboratory Practices and Quality Assurance Requirements: Statements of compliance were provided.

Study Started: September 14, 1995

Study Completed: April 16, 1996

Animals: Male (mean body weight of 3.4 kg; ages were not provided) and female (mean body weight of 2.9 kg; ages were not provided) cynomolgus monkeys.

Methods: Rationale for dosage selection was not provided by the sponsor. Animals were surgically prepared with indwelling cannulas into one of the femoral veins, placed in protective jackets, and connected to infusion pumps. Two groups of 6 monkeys each (3 males and 3 females) were given bolus intravenous injections of r-hirudin (0 and 0.4 mg/kg, respectively), followed by intravenous infusions (0 and 0.15 mg/kg/h, respectively) for 15 days. Vehicle was isotonic sterile saline; infusion rate was 1 mg/kg/h.

Animals were observed at least twice daily for clinical signs of toxicity and mortality. Body weights were recorded once before surgery, twice between surgery and initiation of treatment, on Days 4, 8, 11 and 14 of treatment, and at the end of the observation period (Day 19). Food consumption was estimated daily by the difference between the quantity given and remainder the next morning.

Blood samples were obtained without anesthesia from one of the cephalic or saphena veins for hematology and blood chemistry evaluation in all animals once before beginning treatment, on Day 15 of treatment, and at the end of the observation period (Day 19). Urine samples for urinalysis were collected for at least 14 h during food and water deprivation in all animals once before beginning of treatment and at the end of the observation period (Day 19).

Electrocardiographical examinations were performed on all animals once before the beginning of treatment, on Day 15 of treatment, and at the end of the observation period (Day 19). Systolic and diastolic blood pressures were recorded at the same times.

At the end of the observation period (Day 19), all animals were sacrificed and subjected to a detailed gross pathological examination. Organ weights of adrenals, brain, heart, kidneys, liver, ovaries, pituitary gland, spleen, testes, thymus and thyroids with parathyroids were determined.

Histopathological examinations were performed on the following tissues from all animals: adrenals, aorta, brain, cecum, colon, duodenum, femoral bone, gall bladder, heart, ileum, infusion site, jejunum, kidneys, liver, lungs, lymph nodes, mammary glands, esophagus, ovaries, pancreas, pituitary gland, rectum, salivary glands, spinal cord, spleen, sternum with bone marrow, stomach, testes and epididymides, thymus, thyroids with parathyroids, urinary bladder and uterus.

Blood samples were obtained for assessment of r-hirudin plasma levels on Days 1 through 19 and for assessment of anti-r-hirudin IgG and anti-r-hirudin IgM antibodies on Day 1, 15 and 19. Plasma levels of r-hirudin were determined by

Data were statistically analyzed with analyses of variance and appropriate post-hoc tests.

Results:

1. Observed Effects: There were no treatment-related clinical signs of toxicity.

2. Mortality: There were no deaths.

3. Body Weights: Mean body weights of control males and females on the day before initiation of treatment were 3.2 and 2.8 kg, respectively. Mean body weights of control males and females on Day 18 were 3.2 and 2.9 kg, respectively. There were no treatment-related effects on body weight.

4. Food Consumption: Food consumption was estimated daily by the difference between the quantity given and remainder the next morning. There were no treatment-related effects.

5. Hematology: There were no treatment-related effects.

6. Blood Chemistry: There were no treatment-related effects.

7. Urinalysis: There were no treatment-related effects.

8. Electrocardiography/Blood pressure: There were no treatment-related effects.

9. Organ Weights: There were no treatment-related effects.

10. Gross Pathology: There were no treatment-related effects.

11. Histopathology: There were no treatment-related effects.

12. Plasma Levels of Drug: As shown in the following table for males, plasma levels of r-hirudin were below level of quantitation in controls and ranged in monkeys receiving r-hirudin infusions (0.15 mg/kg/h). r-Hirudin has no effect on IgG titres. One animal (Animal No. M50565) receiving r-hirudin had a mean IgM titre (4.65 µg/ml) that were greater than those in controls

Plasma levels of r-hirudin and antibody titres in males

Animal No.	r-hirudin (mg/kg/h)	Mean plasma level of r-Hirudin (ng/ml)	Mean IgG titre (µg/ml)	Mean IgM titre (µg/ml)
M60561	0	<15.6	<0.94	1.91
M60562	0	<15.6	<0.94	2.55
M60563	0	<15.6	<0.94	2.71
M60564	0.15	403	<0.94	2.38
M60565	0.15	363	<0.94	4.65
M60566	0.15	386	<0.94	2.16

As shown in the following table for females, mean plasma levels of r-hirudin were below levels of quantitation in controls and ranged in 2/3 monkeys (Animal Nos. M60585 and M60586) receiving r-hirudin infusions (0.15 mg/kg/h). One animal (Animal No. M60584) had a mean r-hirudin level of 897 ng/ml that was associated with increases in mean IgG (1.44 µg/ml) and IgM (68.72 µg/ml) titres.

Plasma levels of r-hirudin and antibody titres in females

Animal No.	r-hirudin (mg/kg/h)	Mean plasma level of r-hirudin (ng/ml)	Mean IgG titre (µg/ml)	Mean IgM titre (µg/ml)
M60581	0	15.7	<0.94	2.78
M60582	0	<15.6	<0.94	5.18
M60583	0	<15.6	<0.94	3.10
M60584	0.15	897	1.44	68.72
M60585	0.15	428	<0.94	3.39
M60586	0.15	352	<0.94	2.93

In summary, the no effect dose of intravenously infused r-hirudin in monkeys was 0.15 mg/kg/h. There were no target organs of toxicity. In order to determine organs of toxicity, higher doses would need to be studied. In one female that had relatively high IgG (1.44 µg/ml) and IgM (68.72 µg/ml) titres, mean plasma level of r-hirudin was also relatively high (897 ng/ml), suggesting that antibody formation to r-hirudin might influence the pharmacokinetics of r-hirudin.

One-Month Intravenous Toxicity Study in Monkeys. (Study No. 2549-100)

Study Started: December 28, 1988

Study Completed: June 27, 1989

GLP Requirements: A statement of compliance with the GLP regulations and quality assurance unit was included.

Animals: Young adult cynomolgus monkeys

Drug Batch No.: U002

Methods: Three groups of monkeys (3/sex/group) were administered r-hirudin (dissolved in isotonic saline) at daily i.v. doses of 0.1, 1.0, and 10.0 mg/kg in a volume of 1 ml/kg for a period of 30 days. A separate group of control monkeys (3/sex) received equal volumes (1 ml/kg) of vehicle (sterile saline) alone. Dose selection was based on a dose range finding study in monkeys, wherein r-hirudin was administered at doses of 0.1, 1, 10 and 50 mg/kg i.v. injections for 5 days (except for the 50 mg/kg dose which was only administered for 2 days). R-Hirudin produced no remarkable signs of toxicity except for hematoma formation at the site of blood collection. As a result doses of 0.1, 1.0, and 10 mg/kg were proposed for the 30 day sub acute study in monkeys (Report No. 134-47, Vol. 14, pg 47). Monkeys were examined daily for mortality and clinical signs of toxicity. Body weights were determined at predose and weekly thereafter and food consumption was qualitatively assessed on a daily basis. Ophthalmoscopic exams were conducted at predose and at 2 to 4 hours post dosing on study days 8 and 22. ECG exams (lead I, II, and III) were conducted at predose and on days 9 and 23, following immobilization using ketamine HCl. Hematology and blood chemistry were performed on samples (volume not indicated)

collected twice during the pretreatment period and on study days 3, 10, 17, and 24 (hematology) or on days 10 and 24 (blood chemistry). Ex vivo coagulation parameters were also determined periodically throughout the study. Urinalysis was performed once at pretreatment and on Day 24. Following sacrifice on Days 31 or 32 all monkeys underwent complete gross and histological examinations with determination of organ weights.

Results:

Observed Effects: No treatment-related clinical signs of toxicity were evident. Reddish material was found in the feces of one low dose male but as this was not evident at higher doses it was not considered treatment-related.

Mortality: There were no intercurrent deaths.

Body Weight/Food Consumption: At the end of the treatment period control male and female monkeys weighed 2.7 ± 1.02 kg and 2.2 ± 0.46 kg, respectively. These values were not significantly different from groups treated with r-Hirudin. Similarly, r-hirudin had no adverse effects on food consumption.

Hematology/Bone Marrow: Slight increases in mean APTT values were observed in the 10.0 mg/kg high dose animals (~20% during days 1-27 compared to predose values of 22.7 ± 1.34 sec and 21.4 ± 1.91 sec in males and females respectively. However, control males and females also showed increased APTT values (8 and 9%) over the same time period. In addition, a dose-dependent increase in clotting times (measured at 10 min after dosing on day 30) ranging from in control animals to and >3.5 hours in the 10.0 mg/kg dose groups. Otherwise no treatment-related effects on hematological parameters, bone marrow smears were observed.

Blood Chemistry: No changes in serum chemistry attributable to treatment were observed.

Urinalysis: Urinalysis, findings were limited to a low urinary pH in 2 high dose females and moderate increases in ketone levels (grade 3 or 80 mg/dl) which were observed in all three high dose females as well as one control male, one low dose male and one mid dose female.

Physical Examinations/Ophthalmic and ECG Examinations: Although treatment-related ophthalmoscopic findings (unilateral focal retinopathy, unilateral or bilateral focal pinpoint hemorrhaging and focal de-pigmented spots) were noted in 2 mid dose females (1 mg/kg) and in 2 animals/sex at the high dose (10 mg/kg), beginning on day 22 of the study, the said ocular effects were

not consistently seen in individual animals on repeat examinations and appeared transient (showed signs of resolution) in others. Thus, the aforementioned ocular effects were not of major toxicological concern. ECG analysis in male treated groups revealed mild reductions in heart rates on day 23 relative to control values of 190 b.p.m.) and increased Q-T intervals

However, similar reductions in heart rate and Q-T intervals were observed in all female groups including controls, suggesting that the effects on heart rate and Q-T intervals were not treatment-related.

Organ Weights: No treatment related changes in organ weights were apparent.

Gross Pathology: No treatment-related gross pathological effects were observed.

Histopathology: Microscopic findings of minimal to moderate inflammation, hemorrhage, fibrosis and muscle degeneration at the injection sites were observed in nearly all treated animals. The incidence and severity of the latter was greater in all treated males compared to control males, but was similar to that in control females, except for the following: acute inflammation (1 male each at the 0.1, 1.0, and 10 mg/kg doses) and muscle degeneration (one 10 mg/kg male) and hemorrhage of the skeletal muscle (one 10 mg/kg male) compared to no incidence in either the male or female control groups. In addition, focal retinal hemorrhage was also observed in a mid dose female (#J02188) which presented with similar ophthalmoscopic findings noted earlier. Finally, subacute inflammation of the pancreas was observed in males (one at the 1.0 mg/kg dose and 2 at the 10 mg/kg dose).

Plasma Levels of the Drug: Plasma drug levels were not determined.

In conclusion, administration of r-hirudin to monkeys by bolus i.v. injection (0.1, 1.0, and 10.0 mg/kg) for a period of 30 days was well tolerated, with no mortality or effects on body weights or food consumption observed. However, a dose-dependent increase in clotting times and a slight increase in APTT times (at the 10 mg/kg dose) were observed. In addition, r-Hirudin produced unilateral focal retinopathy, unilateral or bilateral focal pinpoint hemorrhaging and focal depigmented spots in 2 mid dose females and in 2 animals/sex at the high dose, beginning on day 22 of the study. A microscopic correlate of focal retinal hemorrhage was also observed in the mid dose female #J02188 at necropsy. However, the said ocular effects were not consistently seen in individual animals on repeat examinations and appeared transient (showed signs of resolution) in others, and thus were not a major toxicological concern. Other gross and microscopic

findings associated with treatment included: minimal to moderate inflammation, hemorrhage, fibrosis and muscle degeneration at the injection sites. Although the incidence and severity of injection site lesions was greater in all treated males compared to control males, it was similar to that observed in control females, suggesting a possible relationship to the injection technique, rather than r-hirudin per se. Since the focal pinpoint hemorrhagic effects in the retina, observed ophthalmoscopically (two females at the 1.0 mg/kg dose and 2 animals/sex at the 10 mg/kg dose), were apparently transient in nature, and the only other potential dose limiting toxicity involved injection site lesions in males at the 10 mg/kg dose, the no effect dose appears to be between 1 and 10 mg/kg and as such provides an adequate margin of safety for the proposed clinical dose of 0.4 mg/kg.

13-Week I.V. Toxicity Study of r-Hirudin Followed by a 4-Week Recovery Period (Study No. 12507 TCP).

Compliance with Good Laboratory Practices and Quality Assurance Requirements: Statements of compliance were provided.

Study Started: April 5, 1995

Study Completed: December 12, 1995

Animals: Wild-caught male (mean body weight of 3.6 kg; ages were not provided) and female (mean body weight of 2.4 kg; ages were not provided) cynomolgus monkeys.

Methods: According to the sponsor, the low dose of r-hirudin (1 mg/kg/day) for the toxicity study was selected on the basis that it was within the upper range of the intended human dose. Selection of the high dose (30 mg/kg/day) was based upon results obtained in earlier subchronic or reproduction studies (undefined) in monkeys.

Thus, 4 groups of 8 monkeys (4 males and 4 females) each were intravenously administered 0, 1, 10 and 30 mg/kg/day of r-hirudin, respectively, for 13 weeks. Injections were delivered via either the saphena or cephalic veins; injections were alternated between the veins. There were 4 additional monkeys each (2 males and 2 females) in the 0 and 30 mg/kg/day groups that were studied during r-hirudin treatment and a 4-week recovery period. Vehicle was sterile isotonic saline solution; dosing volume was 2 ml/kg.

All surviving animals were observed at least twice daily for clinical signs of toxicity and mortality. Body weights were measured once before initiation of treatment and once a week thereafter, except during week 14 in which body weights were measured twice. Food consumption was estimated daily beginning from one week before initiation of treatment until the end of the study. The quantity of food consumed by each animal was estimated by the difference between the given quantity and the remainder the next morning; actual weights of food consumed were not recorded.

Blood samples for hematology and blood chemistry evaluations were withdrawn from either the saphena or cephalic vein once before initiation of treatment, once on day 8 and once during weeks 4, 13 and 17 in all surviving animals. Blood samples for antibody detection were collected on day 1, during weeks 2, 4, 9, 13, and at the end of the recovery period. Bone marrow samples for myelograms were obtained from the sternum once during weeks 13 and 17.

Urine samples for urinalysis were obtained during day 28 for males, day 29 for females, and day 91 for both sexes in all surviving animals. Samples were collected for the periods of 0-2, 2-4, 4-6, and 6-24 h after dosing.

Electrocardiograms and blood pressure were examined once before initiation of treatment, once on day 30, once on either day 86 or 87, and once at the end of recovery period; ophthalmology examinations were done on the same days. Animals were slightly anesthetized with ketamine hydrochloride (20 mg/kg, i.m.) during these examinations.

Monkeys were terminated after 14 h of fasting by ketamine injection (20 mg/kg, i.m.), followed by thiopental injection (30 mg/kg, i.v.) and exsanguination. Organ weights were determined for adrenals, brain, heart, kidneys, liver, lungs, ovaries, pituitary gland, prostate, spleen, testes, thymus, and thyroids with parathyroids in all animals. All animals were subjected to complete macroscopic examination.

Histopathological examinations were performed for all animals on macroscopic lesions and tissues from adrenals, brain, cecum, colon, duodenum, eyes with optic nerve, femoral bone, gall bladder, heart, ileum, injection sites, jejunum, kidneys, liver lungs, lymph nodes, mammary glands, esophagus, ovaries, pancreas, pituitary gland, prostate, rectum, salivary glands, seminal vesicles, skin, spinal cord, spleen, sternum, stomach, testes and epididymides, thymus, thyroids with parathyroids, tongue, trachea, urinary bladder, uterus, and vagina.

Blood samples for toxicokinetics were withdrawn from either the cephalic or saphena vein in all surviving animals once on day 1, and once during weeks 4, 9, and 13. During day 1, samples were withdrawn at 0.083, 0.25, 0.5, 1, 2, 4, 8 and 24 h after dosing. During weeks 4 and 9, samples were withdrawn at 0.083, 0.25, 0.5, 2 and 24 h after dosing. During week 13 in the 0 and 3 mg/kg/day groups, samples were withdrawn at 0.083, 0.25, 0.5, 1, 2, 4, 8, and 24 h after dosing. During week 13 in the 10 and 30 mg/kg/day groups, samples were withdrawn at 0.083, 0.5, 2, 4, 8 and 24 h after dosing.

Blood samples for antibody detection were withdrawn from either the cephalic or saphena vein in all surviving animals once on day 1 before dosing, during weeks 2, 4, 9 and 13, and at the end of the recovery period.

Normality of variance of data were assessed by the Kolmogorov-Smirnov's test. Subsequently, either analyses of variance or non-parametric tests were applied to the data.

Results:

1. Observed Effects: As shown in the following table (from page 28 of sponsor's Study No. 12507 TCP), the majority of treatment-related clinical signs of toxicity reflected excessive pharmacological properties of r-hirudin.

The major clinical signs which were observed during the treatment period are summarized in the table below:

Dose (mg/kg/day)	0		1		10		30	
Sex	M	F	M	F	M	F	M	F
Swollen areas in different regions	—	—	—	—	—	2/4	2/6	1/6
Decrease in activity	—	—	—	—	—	—	1/6	2/6
Pallor of mucosa	—	—	—	—	—	1/4	1/6	2/6
Thickening at the injection sites	—	—	—	—	3/4	1/4	6/6	2/6
Poor coagulation	—	—	—	1/4	—	2/4	3/6	5/6
Swollen arm or leg (weeks of blood sampling)	—	—	4/4	1/4	4/4	4/4	6/6	6/6
Haematoma	—	—	1/4	—	1/4	—	1/6	1/6
Vomiting/ptyalism	—	—	—	—	—	—	—	1/6
Liquid or soft faeces	—	—	—	—	1/4	1/4	—	—
Wound or crust	1/6	2/6	—	1/4	1/4	2/4	—	1/6

2. Mortality: One male in the 30 mg/kg/day group was found dead on day 41. One female in the 10 mg/kg/day group was sacrificed in a moribund condition on day 77; one female in the 30 mg/kg/day group on day 26; another female in the 30 mg/kg/day group on day 62. All animals had treatment-related bleeding, hematomas and anemia; these toxic effects were considered to be contributing factors to these morbidities/mortalities.

3. Body Weight: Mean body weights of control males were 4.1, 4.5 and 4.6 kg during weeks 1, 13 (last treatment week) and 18 (end of recovery period), respectively. Mean body weights of females were 2.5, 2.8 and 3.0 kg during weeks 1, 13, and 18, respectively. There were no treatment-related effects on body weight.

4. Food Consumption: Food consumption was estimated daily beginning from one week before initiation of treatment until the end of the study. The quantity of food consumed by each animal was estimated by the difference between the given quantity and the remainder the next morning; actual weights of food consumed were not recorded. There were no apparent treatment-related effects on food consumption.

5. Hematology: As shown in the following table, there were treatment-related decreases of hemoglobin; there was complete reversal during the recovery period. There were no other treatment-related effects on hematological parameters.

Hematological effects of r-hirudin in monkeys

Dose (mg/kg/day)		Males			Females		
		1	10	30	1	10	30
Hemo-globin	Day 8	-6%	-2%	-15%	-7%	-2%	-13%
	Week 4	-2%	-5%	-16%	-6%	-24%	-15%
	Week 13	-3%	-1%	-16%	-6%	-12%	-4%

6. Blood Chemistry: There were no treatment-related effects.

7. Urinalysis: There were no treatment-related effects.

8. Electrocardiography/Blood Pressure/Ophthalmology: There were no treatment-related effects.

9. Organ Weights: There were no treatment-related effects.

10. Gross Pathology: As shown in the following table, hematomas were produced by injections per se (control group had hematomas) and by r-hirudin (incidence and severity was higher in treated groups versus control groups). On the other hand, indurations were not produced by injections per se (control groups had none), but were produced by r-hirudin (treated groups had indurations). These treatment-related effects on injection sites were not seen at the end of the 4-week recovery period.

Combination scores* reflecting incidence and severity of injection-site hematomas and indurations

Dose (mg/kg/day)	Males				Females			
	0	1	10	30	0	1	10	30
Hematomas*	0.19	0.29	0.44	0.25	0.15	0.19	0.38	0.46
Indurations*	0	0	0.02	0.15	0	0.02	0.06	0.29

*Total number of incidences x mean severity score divided by levels of severity (3) x number of animals (4) per vein (2) per sex (2) per group (= 48).

Furthermore, 1/4 males in the 30 mg/kg/day group had a hematoma on the surface of the left kidney at the end of the treatment period.

11. Histopathology: As shown in the following table, hemorrhages and hemosiderosis were produced by injections per se (control group had hemorrhages and hemosiderosis) and by r-hirudin (incidence and severity was higher in treated groups versus control groups).

Combination scores* reflecting incidence and severity of injection-site histopathological lesions (hemorrhages and hemosiderosis)

Dose (mg/kg/day)	Males				Females			
	0	1	10	30	0	1	10	30
Hemorrhage*	0.47	0.60	0.91	0.95	0.63	0.67	1.20	1.63
Hemo-siderosis*	0.21	0.23	0.93	0.90	0.38	0.50	1.43	0.73

*Total number of incidences x mean severity score divided by levels of severity (3) x number of animals (4) per vein (2) per sex (2) per group (= 48).

Furthermore, there was moderate bilateral retinal hemorrhage and minimal unilateral periocular hemorrhage in 1/4 males in 30 mg/kg/day group; moderate to marked hemorrhage and/or encapsulated hematomas in subcutaneous tissues and/or skeletal muscle of hindlimbs in 2/4 females in the 10 mg/kg/day group and 2/4 males and 2/4 females in the 30 mg/kg/day group; and paracapsular encapsulated hematoma on left kidney of 1/4 males in 30 mg/kg/day group.

Finally, as shown in the following table, the incidence and severity of red bone marrow increased as a function of dose.

Incidence and severity of red (hemopoietic) bone marrow

Dose (mg/kg/day)	Males				Females			
	0	1	10	30	0	1	10	30
Minimal to slight red bone marrow	2/4	1/4	1/4	3/4	2/4	1/4	2/4	2/4
Moderate to severe red bone marrow	0/4	0/4	0/4	1/4	0/4	0/4	2/4	1/4

All histopathological lesions were less severe or completely absent at the end of the 4-week recovery period.

12. Plasma Levels of Drug: As shown in the following table, there were dose-related increases in mean C_{max}s and AUCs. At higher doses, AUCs were greater on Day 89/90 than on Day 1. The other toxicokinetic parameters ($t_{½\alpha}$, $t_{½\beta}$, V_{Dss}, CL) remained relatively stable among doses and between Day 1 and Day 89/90. Data were pooled for males and females because there were no apparent differences.

Toxicokinetic parameters for intravenously administered r-hirudin in monkeys

Pharmaco-kinetic parameter	1 mg/kg/day		10 mg/kg/day		30 mg/kg/day	
	Day 1	Day 89/90	Day 1	Day 89/90	Day 1	Day 89/90
C_{max} (ng/ml)	9691	7911	78741	64496	226180	218965
$t_{½\alpha}$ (h)	0.17	0.14	0.13	0.13	0.12	0.13
$t_{½\beta}$ (h)	3.22	2.01	1.86	3.08	1.79	2.07
V_{Dss} (l/kg)	0.52	0.38	0.40	0.43	0.41	0.39
CL (ml/min/kg)	3.73	4.38	5.23	6.79	5.73	4.50
AUC (ng/ml•h)	4618	4143	33112	73709	95624	119498

Furthermore, a relationship between antibody formation to r-hirudin and r-hirudin AUCs became evident in further analysis of the data; methodology for antibody detection and quantitation was not provided by the sponsor. As shown in the following table, there were dose-related increases in AUCs. Furthermore, AUCs were higher in animals in which antibodies for r-hirudin were detected in the blood by day 89/90 than in animals in which no antibodies were detected. There was also a positive correlation between AUCs and APTTs.

Effects of antibody formation to r-hirudin on AUCs for r-hirudin

Dose (mg/kg/day)	Day	No Antibodies AUCs (ng/ml•h)	Antibodies Present by Day 89/90 AUCs (ng/ml•h)
1	1	4123	5277
	89/90	3209	5700
10	1	33956	32268
	89/90	28623	118796
30	1	89476	100015
	89/90	95256	159902

13. APTT Trough Levels on Day 85 (Amendment Submitted to the report 12507 TCP, page 284, volume 1.37): The APTT trough levels were increased in the presence of antibodies particularly in high dose groups.

APTT Trough Levels on Day 85 in Monkeys			
Dose (mg/kg/day)	Antibodies Present By Day 89/90	APTT on Day 85 (sec)	
		N	Mean ± SD
1	No	5	21.6 ± 0.8
	Yes	3	24.8 ± 2.3
10	No	4	23.0 ± 2.9
	Yes	3	31.0 ± 4.6
30	No	5	21.5 ± 0.6
	Yes	4	31.6 ± 5.4

14. Effect of r-Hirudin Antibodies on the Pharmacokinetic Behavior of the Drug (Amendment Submitted to the Report 12507 TCP, Page 290, Volume 1.37): The median results of the pharmacokinetic analysis of all three dose groups up to 8 hr were as follows:

Summary of pharmacokinetic results on Day 1 and Day 89/90
- Median values -

	Day 1			Day 89/90		
	Antibodies by Day 89/90:			Antibodies by Day 89/90:		
	No	Yes	Total	No	Yes	Total
Number of animals	16	14	30	13	9	22
Initial half-life (hours)	0.13	0.13	0.13	0.12	0.16	0.12
Terminal half-life (hours)	1.87	1.89	1.87	1.28	3.39	2.26
Mean residence time (hours)	1.35	1.30	1.30	0.89	3.51	1.43
Total clearance (ml/min/kg)	4.95	5.15	5.04	5.24	2.76	4.85
Volume of distribution at steady-state (L/kg)	0.40	0.40	0.40	0.28	0.50	0.37

Data indicated that on day 89/90, plasma clearance of r-hirudin decreased and $t_{½}$ ß was increased in monkeys which had r-hirudin antibodies.

Thus, the minimal effect i.v. dose of r-hirudin in a 13-week toxicity study in monkeys was 1 mg/kg/day. The 30 mg/kg/day dose produced mortality in one male, and the 10 and 30 mg/kg/day doses produced a moribund condition requiring sacrifice of 3 females. The 10 and 30 mg/kg/day doses also produced treatment-related decreases in hemoglobin, treatment-related hematomas and indurations at injection sites, and microscopic hemorrhages and hemosiderosis at injection-sites. These effects reflect exaggerated pharmacological activities and were less severe or completely absent at the end of the 4-week recovery period. There were dose-related increases in C_{max}s and AUCs; AUCs reflected accumulation of plasma r-hirudin over 90 days. Antibody formation to r-hirudin was associated with increased AUCs and APTTs.

3-Month Subcutaneous Toxicity Study with r-Hirudin in Monkeys. (Study No. 90.0762)

Testing Laboratories: Pharma Research, Toxicology and Pathology
Hoechst AG, D-6203 Frankfurt(M) 80

Study Started: June 13 to 16, 1989

Study Completed: July 20, 1990

GLP Requirements: A statement of compliance with the GLP regulations and quality assurance unit was included.

Animals: Rhesus Monkeys; about 4 years of age,

Drug Batch No.: C 003, C 009

Methods: Groups of Rhesus monkeys (4/sex/group) were subcutaneously administered either vehicle (0.9% NaCl) or r-Hirudin at doses of 0.15, 1.5, and 15 mg/kg, administered in total volumes of 0.1 ml/kg twice daily for 90 days (i.e. total daily dose = 0.30, 3.0, and 30 mg/kg). One monkey/sex in the control, 0.15, and 1.5 mg/kg groups and one male at the 15.0 mg/kg dose level underwent an additional 4 week recovery period. The 15 mg/kg high dose (approximately 100 times the expected therapeutic dose) was selected based on a preliminary 25 day repeat-dose s.c. study in monkeys in which r-Hirudin, at a dose of 15 mg/kg twice daily, lead to prolongation of coagulation parameters (coagulation-time, APTT, PT, and TT), blood

infiltration at the injection sites, and a "latent danger of bleeding". Clinical signs of toxicity and food and water consumption were determined daily, with body weights determined weekly. Neurological evaluations (including hearing) were performed at predose and every 14 days during treatment. Ophthalmoscopic and ECG exams were conducted prior to dosing, after 5-weeks of treatment (high dose animals only), at the end of the study, and at the end of the recovery period (recovery animals only). Hematological, clinical chemistry and urinalysis were conducted on samples collected at predose, at 1 week (urinalysis only) and one month after the start of treatment, at the end of the treatment period, and at the end of the recovery period. (Note: blood samples [volume not indicated] were collected approximately 15-17 hours after the preceding p.m. dose and urine samples were collected overnight). Test for occult blood in the feces were performed prior to treatment, at 1-2 weeks, 1 month and 2 months after the start of treatment and at the end of the treatment period. Plasma levels of test article were determined in one monkey/sex/group prior to and at 0.5, 1, 2, 4, and 7 hours after the first and 85th a.m. dose and 16 hours after the first and 85th p.m. doses. Humoral hirudin antibodies were also tested for in serum samples collected prior to dosing, at 5 weeks after dosing and at the end of the treatment period. Monkeys which died, or which were sacrificed at the end of the treatment or recovery periods underwent complete gross and histological examinations with determinations of organ weights including determinations of organ weights.

Results:

Observed Effects: Subcutaneous administration of r-Hirudin in monkeys induced a dose-dependent increase subcutaneous bleeding at the injection sites and red discoloration of the skin at all doses, with subcutaneous thickening of the skin also observed at the 1.5 and 15 mg/kg doses. Animals at the 15 mg/kg dose also showed extensive blood infiltrations and hematomas at the injection site and/or other parts of the body and, single instances of severe acute bleeding days (three high dose females). Finally, periodic signs of anemia: pale mucous membranes, apathy, and/or weakness were observed in females at the high dose. Test for fecal occult blood were positive in individual animals form all groups including controls, with no evidence of a treatment-related effect on the incidence.

Mortality: Severe acute bleeding, resulted in the death of one female on day 25. In addition one 25 mg/kg dose male was sacrificed prematurely on day 89 due to extreme exophthalmus induced by edema in the orbit which probably occurred secondary to a hematoma in the skull.

Body Weight/Food Consumption: Periodic reductions in food and water consumption occurred in 2 of 4 males and all females at the high dose. Body weights for control monkeys increased slightly (3-15%) over the treatment period and averaged 4.075 ± 0.475 kg and 4.275 ± 0.330 kg in males and females at the end of the treatment period, respectively. Treatment with r-hirudin at the 15 mg/kg dose resulted in no net gain in one high dose male and in body weight losses of 0.1, 0.2 and 0.6 kg in three of the high dose females over the same period. The 0.6 kg loss of body weight occurred from day 22 to the time of death on day 25 and was attributable to a lack of food and water consumption during the said time period.

Hematology/Bone Marrow: Hematologic findings at the end of the period included: a dose dependent increase in coagulation time, APTT, and TT at all doses, with increased PT also observed at the 1.5 and 15 mg/kg doses. The maximal anticoagulative activity of r-Hirudin was observed at 3 hours after the a.m. dosing, but was still present at the two high dosages, 16 hours after the p.m. administration and was more marked and longer lasting after repeated administration compared to the first dose. No other hematological effects were observed at the 0.15 and 1.5 mg/kg doses. At the 15 mg/kg high dose, both males and females showed anemic effects (i.e. reductions in erythrocytes (17 and 38%), hemoglobin (20 and 46%) and hematocrit (20 and 43%), relative to values of 6.55 and 6.49 x 10^9 cells/ml, 142 and 138 g/dl, and 50 and 49%, in control males and females, respectively. In general, anemic effects were more severe in females versus males, with severe microcytic anemia observed in one high dose female at the end of the study (i.e. large reductions in erythrocytes, 67%; hemoglobin, 79%; and hematocrit, 76%). Increased reticulocyte numbers were also observed in high dose males and females (2.6 fold in each sex, relative to control values of 1.6 and 2.2% in males and females, respectively). Finally, 1 of 4 high dose males and 2 of 4 high dose females showed increased thrombocyte numbers (60% to 2.6 fold, relative to control values of 342 and 438 x 10^9/l in males and females, respectively). Examination of the bone marrow also revealed increased hematopoiesis in 2 females at the 0.15 mg/kg low dose and in 2 males and 3 females at the 15 mg/kg high dose, but in none of the mid dose animals.

Blood Chemistry: Clinical chemistry findings at the end of the study included: a slight decreases in serum cholesterol and reduced serum sodium (12%), calcium (18%) and magnesium (29%) in females at the 15 mg/kg high dose. However, the biological significance of the aforementioned findings is not apparent.

Urinalysis: Urinalysis showed no treatment-related changes indicative of metabolic changes or organ damage.

Physical Examinations/Ophthalmic Examinations/ECG Examinations: Ophthalmologic findings were limited to a marked anemic fundus in 1 high dose female, with no treatment-related effects on ECG parameters noted.

Organ Weights: Increased absolute and relative weights for heart were noted in both male (15.4 and 15.6%) and females (17.8 and 24.3%), respectively. Organ weights for heart remained elevated in both sexes at the end of the recovery period.

Gross Pathology: No treatment-related gross observations were observed in monkeys at the 0.15 and 1.5 doses except for an increased incidence and severity (up to 4 cm in diameter) subcutaneous patches of red discoloration at the injection site of all animals at the aforementioned doses versus 2 of 6 control animals. At the 15 mg/kg dose, red patches involved large areas (up to 10 to 20 cm in diameter) of the subcutis, were bloody and gelatinous, and showed partial induration of the connective tissues. Hemorrhagic effects at areas other than the injection site were also observed in animals at the 15 mg/kg dose [i.e. thymus: red discoloration, red tissue induration, and hematomas (males #25 and #27, and female #20); underneath the right kidney and in the peritoneal layer of the rectum (male #27); urinary bladder (males #25 M and #27 and female #32); esophagus, stomach, mucus membrane of the distal colon, and rectum wall (female #30); musculature of the thigh and/or lower leg (females #30 and #32)]. Female #31 also showed pale liver and kidneys. Finally, the 15 mg/kg female #32 which died had extended arachnoidal bleeding in the frontal lobe of the cerebrum, hemorrhage of the subperitoneal tissue and mesentery and red speckles on the visceral side of the urinary bladder, swollen spleen, and pale liver, kidney, pancreas and heart as well as edema and emphysema of the lungs.

Histopathology: Subcutaneous injection of r-Hirudin in monkeys for 3 months, resulted in a dose-dependent increase in incidence and severity the hemorrhage/necrosis and subcutis or perivascular round cell aggregation at the injection sites (Site #1 and Site #2). In addition, individual monkeys at the high dose also showed stages of secondary organization hemorrhage and/or hematoma at the injection site.

Histological effects other than lesions at the injection site were mainly limited to monkeys at the 15 mg/kg high dose, namely the female (#32) which died on Day 25 and the male (#25) which was sacrificed on day 89. The said female (#32) showed histological findings of: hemorrhage in the brain (cerebrum, cerebellum, and medulla oblongata), eye (retinal hemorrhage and round cell aggregation), and skeletal muscle (head and face); hyperemia of the spleen; and bone marrow hyperplasia. The high dose male (#25) which was sacrificed on day 89 also showed hemorrhage in the urinary bladder, thymus, and skeletal muscle;

round cell aggregation and inflammation of the fornix of the eye and bone marrow hyperplasia. Treatment-related effects in other 15 mg/kg male (M) and female (F) monkeys which survived to the end of the treatment period included hemorrhage in the following organs: kidney (#27 M), urinary bladder (#27 M); thymus (#27 M and #30 F); stomach (#30 F), rectum (#27 M and #30 F), esophagus (#30 F), trachea (#30 F), aorta (#30 F), skeletal muscle of the head (#29 F and #30 F), diaphragm (#30 F), and skin (#26 M). In addition, round cell aggregation of the eye (#29 F and #30 F) and bone marrow hyperplasia (#27 M, #29 F, and #30 F) were also observed at the high dose. Bone marrow hyperplasia was also observed in 2 low dose females. However its relationship to treatment is unknown, since none of the mid dose monkeys showed this effect. Increased heart weights in the high dose animals had no histological correlates. Histological findings which remained in monkeys which underwent the recovery period included: hemorrhagic effects in the esophagus and aorta of the 15 mg/kg recovery male, and secondary organizational hemorrhage and/or round cell aggregation at the injection sites (1.5 mg/kg male and female recovery animals and the 15 mg/kg recovery male).

Plasma Levels of the Drug: Mean maximum blood levels of r-Hirudin (measured in one male and female/dose group) increased in a linear fashion with increasing dose and occurred between 1 and 2 hours after the morning dosing on both day 1 and day 85 of the study. Maximal plasma levels on day one were 174 and 206 ng/ml; 1202 and 1233; and 9513 and 13112 ng/ml at the 0.15, 1.5, and 15 mg/kg doses, respectively. On Day 85, plasma concentrations of r-Hirudin were increased to 2807 and 1021 ng/ml; 3268 and 6739 ng/ml; and 12371 and 30338 ng/ml at the at the 0.15, 1.5, and 15 mg/kg doses, respectively. In addition, predose plasma levels of 1461 and 292 ng/ml; 428 and 2322 ng/ml; and 568 and 5490 ng/ml were observed in the male and female tested on day 85. Plasma levels were still present 7 hours after the a.m. and 16 hours after the p.m. administrations.

In conclusion, subcutaneous administration of r-Hirudin at doses of 0.15, 1.5, and 15 mg/kg produced no clinical signs of toxicity except for a dose-dependent increase in the incidence of injection site lesions (hemorrhage and/or round cell aggregation; mainly at the mid and high doses) and hemorrhages in other areas of the body (at the 15 mg/kg high dose). Administration of the 15 mg/kg dose produced excessive hemorrhagic effects, including internal organ hemorrhage which resulted in the death of one female (day 25) and the sacrifice of a male on day 89. The immunogenic potential of r-Hirudin was evaluated but the results were not currently reported. These results should be submitted to the agency for complete review. No specific target organs of toxicity were identified, except for a dose-dependent inhibition of hemostasis mechanisms. With the exception of injection site lesions (moderate to marked, hemorrhage/necrosis and round cell aggregation), the 1.5 mg/kg dose was well tolerated and could be considered the no effect dose for the study.

REPRODUCTIVE TOXICITY:

I.V. Segment I. Fertility and General Reproductive Performance Study in Rats
(Study # 12505RSR)

Study Started: March 9, 1995

Study Completed: December 7, 1995

GLP Requirements: A Statement of Compliance with GLP regulations was included.

Animals: Crl:CD(SD)BR Sprague Dawley rats (males: 40 days old, about 200 g and females: 10 weeks old, about 257 g).

Drug Batch No.: 115011

Methods: Groups of rats (30 rats/sex/group) were given i.v. doses of 0 (vehicle: 0.9% saline), 1, 10 and 30 mg/kg/day of r-hirudin. The volume of administration was fixed at 1 ml/kg. The male rats were treated from 64 days prior to mating throughout the mating phase and until they were sacrificed. Females were treated from 15 days prior to mating throughout mating and up to day 7 of gestations. Six rats/sex/group were used for toxicokinetic study. Sponsor indicated that "low dose-level (1 mg/kg) is within the upper range of the intended human dose. The high dose-level (30 mg/kg) is about 100-fold higher than the intended dose-level for different clinical conditions". All rats were observed daily for clinical signs and mortality. Body weights and food intakes were recorded weekly. All dams were sacrificed on day 15 of gestation and were examined for the number of corpora lutea, the number of implants and number of live/dead fetuses. Males were sacrificed after the first week of hysterectomy of the females. Male sperm were sampled from the caput epididymis and analyzed for number and viability of spermatozoa. Males and females both were subjected to complete macroscopic examinations. On day 1 and on day 63 of the study, blood samples were collected at pre-dose, 15 min, 1, 4 and 8 hr after drug administration for monitoring drug levels in plasma.

Results: One male from mid dose group and one male from high dose group were found dead during study period. The cause of death could not be established. Mating and fertility of male rats were unaffected by the treatment. r-Hirudin had no effect on sperm morphology nor had any effect on estrous cycle. No treatment related effects were seen on number of corpora lutea, number of implants, number of live and dead concepti.

Mating and Fertility of F_0 Male Rats				
Parameters	**Control**	**Low Dose**	**Mid Dose**	**High Dose**
# of Male Pairs	24	24	24	23
# of Males Mated	23	23	24	23
Mating Rate (%)	95.8	95.8	100.0	95.8
# of Pregnant	22	22	24	22
Male Fertility Index (%)	95.7	95.7	100.0	95.7

# of male fertility index = (# of pregnant females/# of mated males) x 100

Dams Sacrificed on Day 15 of Pregnancy				
Parameters	**Control**	**Low Dose**	**Mid Dose**	**High Dose**
# of Pregnant Dams	23	23	24	23
# of Corpora Lutea	409	406	434	407
# of Corpora Lutea/Dam	17.8	17.7	18.1	17.7
# of Implants	379	340	385	356
# of Implants/Dam	16.5	14.8	16.0	15.5
Pre-implantation Loss (%)	7.3	16.3	11.3	12.5
Post-implantation Loss (%)	0.7	1.0	0.9	0.8
Live Fetus/Dam	15.8	13.7	15.2	14.7
Dead Fetus/Dam	0.7	1.0	0.9	0.8

In conclusion, there were no abnormal effects on the fertility and mating performance of the treated male and female rats at i.v. doses up to and including 30 mg/kg/day of r-hirudin.

Two Phase I.V. Segment II Teratology Study with R-Hirudin in Rats
(Report No. 9583 RSR)

Study Started: January 26, 1993

Study Completed: January 20, 1994

GLP Requirements: A Statement of compliance with the GLP regulations and quality assurance unit was included.

Animals: Sprague Dawley mated female rats, 9-10 weeks of age, (mean body weight = 250 g).

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ON ORIGINAL

Drug Batch No: 20

Methods: Four groups of inseminated female rats (36/group) were administered r-Hirudin (dissolved in isotonic saline) intravenously at doses of 0 (vehicle), 1, 10, and 30 mg/kg/day in a total volume of 1.0 or 1.5 ml/kg on gestation days 7 through 17 inclusive. The basis of dose selection was not indicated. Clinical signs and mortality were checked daily. Food consumption and body weights were determined periodically throughout pregnancy and up to day 21 postpartum. On day 20 of pregnancy 2/3 of the F0 females (24 females/group) were sacrificed, examined macroscopically, and the fetuses were removed by Caesarian section and litter parameters (number of corpora lutea, number and distribution of live and dead fetuses, number and distribution of early and late resorptions, and the number of implantation sites) were determined. All live F1 fetuses were weighed, sexed, and examined for external malformations. Half of the F1 fetuses underwent soft tissue examinations and the other half underwent skeletal examinations for identification of anomalies and malformations. The remaining 1/3 of the F0 females were allowed to deliver and rear their progeny until the time of weaning (postpartum day 21). Litter size and weights and any gross abnormalities were noted for F1 pups in the delivery and wean subgroup. Litters from the Delivery and Wean subgroups were culled to 4 pups/sex/litter on postpartum day 4. Culled F1 pups were examined daily for clinical signs and the onset of development parameters were recorded. On Day 21, one animal/sex/litter (12 rats/sex/group) was selected and monitored daily for mortality and clinical signs with food consumption and body weights determined weekly. Between 6 and 8 weeks of age, F1 offspring were examined for effects on learning and memory, spontaneous activity, and auditory function. At 10 to 11 weeks of age, selected F1 offspring were then mated and allowed to deliver normally. Each F2 litter was examined for determination of litter size, number

of live, dead or cannibalized pups, and pup weights. F2 pups were culled to 4 pups/sex/litter on postpartum day 4. Culled pups were monitored daily for clinical signs and the onset of development parameters were recorded. Following sacrifice, all parental animals (F0 and F1 generations), and all pups (F1 and F2, including any which died during lactation) underwent gross internal examinations.

Results: Mean maternal and fetal parameters in for females in the term sacrifice subgroup are presented in Table 7, on the following page.

Table 7. F0 Maternal and Litter Parameters in the Term Sacrifice Subgroup, Segment II Reproductive Toxicity Testing in Rats

Treatment	Vehicle	r-Hirudin (mg/kg)		
Dose (mg/kg)	0	1	10	30
Parameter measured:				
F0 Maternal data				
# gravid rats	22	20	21	21
# Total Resorptions	1	0	0	0
# Aborted	0	0	0	0
# Early Deliveries	0	0	0	0
# Which Died	0	0	0	0
Mean Weight Δ (g)[a]	179 ± 25	189 ± 23	182 ± 23	174 ± 28
F1 Fetal data				
# Corpora lutea/doe[am]	16.6 ± 4.0	17.7 ± 1.7	16.1 ± 2.0	16.1 ± 3.0
# implantations/doe[am]	13.5 ± 4.1	15.2 ± 1.6	14.1 ± 2.3	13.5 ± 3.9
# resorptions/doe[am]	0.6 ± 1.1	0.4 ± 0.7	0.8 ± 1.1	0.8 ± 0.8
# living pups/doe[am]	13 ± 4.7	14.8 ± 1.7	13.3 ± 2.9	12.7 ± 3.9
# dead pups/doe[am]	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Mean litter size	13 ± 4.7	14.8 ± 1.7	13.3 ± 2.9	12.7 ± 3.9
Mean Fetal Weights	3.96 ± 0.28	4.0 ± 0.24	4.02 ± 0.31	4.12 ± 0.35
% Preimplantation loss[b]	18.4	14.1	12.1	16.3
% Postimplantation loss[c]	4.4	2.6	5.7	5.7

[a] -Body weight gains days 2 through 20 for all females in the term sacrifice and delivery and wean subgroups.
[b] -[(# corpora lutea - implant sites)/corpora lutea] x 100
[c] -[(#implant sites - viable fetuses)/implant sites] x 100

Treatment with r-Hirudin produced no mortalities, abortions, or clinical signs of toxicity. In addition, no treatment-related effects on food consumption or body weight gains were observed. Further, there were no treatment-related effects on the mean numbers of corpora lutea, implantation sites, live fetuses, post implantation loss (resorptions or dead fetuses) or mean fetal body weights. Slight variations in the sex ratio were observed, but were not dose related and thus not considered treatment-related.

Gross examinations of F1 fetuses for external malformations yielded a single instance of coelosomy in one of 267 fetuses examined (0.4%) in 1 of 21 litters (4.8%). This was not considered treatment-related. The other 285, 296, and 280 fetuses in control and 1 and 10 mg/kg/day r-hirudin groups had no external malformations. Observed soft tissue anomalies were limited to a low incidence of dilated renal pelvis (3, 3, 5, and 6 fetuses), ureteral dilation (2, 0, 3 and 2 fetuses) and blood in the thoracic cavity (0, 0, 1, and 0 fetuses) and ventricular cerebral dilations of the brain (0, 0, 1, and 0 fetuses) of the 137, 143, 136, and 127 fetuses examined at the 0, 1, 10, and 30 mg/kg doses, respectively. The low and non dose-dependent incidence of the aforementioned effects suggested that they were not treatment-related. No skeletal malformations were observed in fetuses from groups treated with r-hirudin. Table 8 below depicts some of the more prevalent skeletal variations and anomalies which were observed.

Table 8. Incidence of Skeletal Variations and Anomalies in F_1 Fetuses from a Segment II Reproductive Toxicity Testing in Rats.

Treatment Dose(mg/kg)	Vehicle 0	r-Hirudin 1	 10	 30
Number of Litters Evaluated	21	20	21	21
Number of Fetuses Evaluated	148	153	144	140
	No. of Fetuses/No. of Litters Affected			
Skeletal Variations				
Sternebra (5th or 6th Sternebra) Reduced Ossification/Unossified.....	107/20	120/19	108/21	104/19
Unossified 4th Metacarpal.....	2/2	5/5	2/2	1/1
Skeletal Anomalies:	5/3	9/4	8/5	8/6
Skull (Hyoid) Reduced Ossification.....	8/5	12/7	6/5	12/8
	2/2	2/2	2/1	1/1
Unossified.....	0/0	1/1	1/1	0/0
Thoracic Vertebra Reduced Ossification.....	5/5	7/5	10/3	6/5
	0/0	0/0	0/0	1/1
Unossified.....				
Sternebra (1st to 4th Sternebra) Reduced Ossification/Unossified.....				
Ribs (Reduced Ossification).....				

Note: Variations/anomalies, both refer to non-permanent structural changes which have no obvious detrimental effects. Changes classified as variations occurred at a > 10% incidence in the historical population. Finally, variations/anomalies which occurred only at the mid or low doses or which occurred at a greater incidence in the control groups are not depicted above.

Briefly the data presented in Table 8 shows that, r-Hirudin treatment produced no treatment-related effects on the incidence of skeletal variations or anomalies in the F1 fetuses in the term sacrifice subgroup which were examined.

In the subgroup which was allowed to deliver, the period of gestation was within the normal range for all groups and all pups were born alive. A tabulated summary of the maternal and litter data form the sub group which was allowed to deliver is presented in Table 9 below:

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Table 9. **F0 Maternal and Litter Parameters in the Term Sacrifice Subgroup, Segment II Reproductive Toxicity Testing in Rats**

Treatment	Vehicle	(r-Hirudin)		
Dose (mg/kg)	0	1	10	30
Parameter measured:				
F0 Rat data				
# gravid rats	12	12	12	12
# aborted	0	0	0	0
# early deliveries	0	0	0	0
# which died	0	0	0	0
Mean Weight Δ (g)[a]	179 ± 25	189 ± 23	182 ± 23	174 ± 28
Litter data				
Mean litter size	13.9 ± 3.7	15.8 ± 1.3	14.7 ± 1.9	14.6 ± 2.3
# living pups/doe am	13.9 ± 3.7	15.8 ± 1.3	14.7 ± 1.9	14.6 ± 2.3
# dead pups/doe am	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
Sex Ratio (Male Pups/total Pups) %	52.1	52.4	52.3	56.0
Pup Weight/litter g[b]	6.4 ± 0.5	6.4 ± 0.2	6.8 ± 0.6	6.9 ± 0.7
Pup Weight/litter g[c]	52.6 ± 4.5	52.6 ± 4.5	52.6 ± 4.5	52.6 ± 4.5

TA

[a] -Body weight gains days 2 through 20 for all females in the term sacrifice and delivery and wean subgroups.
[b] -Pup Weight/litter on day 1
[c] -Pup Weight/litter on day 21

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The pup viability indices (Number of surviving pups/Number of live born pups) X 100 on postpartum day 4 and 21 were similar between control and treated groups (range between 95 and 99%), with no treatment-related effects observed. In addition, no treatment-related effects on the onset of physical development parameters (pinna unfolding, hair growth, tooth eruption, eye opening, and auditory canal opening), learning and memory tests were observed in the F1 offspring. Similarly, treatment with r-hirudin had no effects on either reflex development (surface righting, cliff avoidance, and air righting) or behavioral parameters (learning and memory maze tests, the open field test

for spontaneous activity and the startle reflex test) and produced no clinical effects attributable to treatment. F1 pups (both sexes) from treated F0 females also showed no effects on food consumption or body weight gains relative to control gains of 418 and 236 g in males and females (postnatal days 1-50). Body weight gains and food consumption in mated F1 females from treated groups were not significantly different from control values during the periods of gestation and lactation, except for a slight increase in body weight gains (16%) at the 10 mg/kg mid dose during gestation days 0-20; relative to control gains of 185 g. However, since this effect was limited to the mid dose it was not considered treatment-related. Analysis of the F1 reproductive data showed that 2 of the 12 pairs of rats in each of the treated groups were not able to mate. This resulted in a slightly reduced mating index (83%) relative to 100% in control animals. However, females which were mated showed fertility indexes of 100% in all groups except at the mid dose where the fertility index was 90%. In addition, the mean number of implantation sites was similar in the control (17.3), 1 (16.8), 10 (17.9), and 30 mg/kg/day groups and no effects on the gestation index (100% in all cases) or the duration of gestation observed.

Although none of the F2 pups were stillborn, two entire F2 litters one each at the 1 and 10 mg/kg doses died resulting in a lower viability index for both groups (79.9 and 65.8%, respectively), relative to indices of 87.0 and 94.6% in control and the 30 mg/kg dose. However, the litter deaths at the 1 and 10 mg/kg doses were considered equivocal since no such effect were seen at the high dose. Finally the F2 offspring showed no treatment-related effects on pup body weights, developmental and reflex parameters (same as for F1 above), clinical signs or on macroscopic findings attributable to treatment. No treatment-related macroscopic changes were noted in F2 pups which were killed at the end of the study, whereas macroscopic changes in the pups which died could not be noted due to autolysis.

In conclusion, administration of r-Hirudin intravenously at doses of 1, 10, and 30 mg/kg/day during the period of organogenesis was well tolerated at all doses tested and was neither embryotoxic nor teratogenic and did not impair the development, behavior or reproductive capacity of the offspring. The 30 mg/kg high dose was the no effect dose for the current study in rats and is 30 fold greater than the intended human dose of 1 mg/kg. However, as was noted previously, no basis of dose selection was indicated, and thus, the appropriateness of dose selection for the study could not be determined.

I.V. Segment II Teratology Study with R-Hirudin in Rabbits
(Report No. 9584 RSL)

Study Started: January 25, 1993

Study Completed: November 18, 1993

GLP Requirements: A Statement of compliance with the GLP regulations and quality assurance unit was included.

Animals: Mated New Zealand White Hy/Cr mated female rabbits; approximately 16-18 weeks of age; Mean body weights = 3.5 kg.

Drug Batch No. 20 and 19

Methods: Four Groups of inseminated female rabbits (16/group) were administered r-Hirudin (dissolved in isotonic saline) intravenously at doses of 0 (vehicle), 1, 10, and 30 mg/kg/day in a total volume of 0.5 ml/kg on gestation days 6 through 18 inclusive. The basis of dose selection was not indicated. Mortality, signs of morbidity, and clinical signs (including signs of abortion) were checked daily. Food consumption and body weights were determined periodically up to day 28 of the pregnancy. On day 28 of gestation, F0 females were killed and the fetuses were removed by Caesarean section. The reproductive tract complete with ovaries was dissected out and the number of corpora lutea, the number and distribution of live and dead fetuses, the number and distribution of early and late resorptions, and the number of implantation sites were determined. Dams were also subjected to macroscopic examinations. Finally, all fetuses were examined for external gross malformations and body weights were determined for live fetuses. Live fetuses were also sexed and examined for internal soft tissue anomalies and malformations and for skeletal variations, anomalies, and malformations.

Results: No clinical signs were observed in the control or 1 mg/kg/day dose groups. However, treatment-related clinical signs of bleeding at the injection sites was noted in 15 of 16 and all 16 rabbits dosed with the 10 and 30 mg/kg doses, respectively. At the 10 mg/kg/day dose, one female was sacrificed on day 7 in poor clinical condition following a fracture to the spinal column, while another female was found dead on day 7, with death attributable to an intercurrent pulmonary pathology (Neither death was considered treatment-related). In addition, no abortions occurred in any group including controls. Control rabbits gained an average of 524 grams from Day 2 through 28 of pregnancy, with comparable gains in body weights and no

differences in food consumption observed in r-hirudin-treated groups, relative to controls. Table 10 below, provides a tabulated summary of the effects of r-Hirudin treatment on maternal and fetal parameters observed in the study.

Table 10. F0 Maternal and Litter Parameters in the Segment II Reproductive Toxicity Study in Rabbits

Treatment	Vehicle	(r-Hirudin)		
Dose (mg/kg)	0	1	10	30
Parameter measured:				
F0 Maternal data				
# gravid rabbits	15	15	15	15
# Total Resorptions	0	0	0	0
# Aborted	0	0	0	0
# Does Which Died	0	0	2	0
Mean Weight Δ (g)[a]	524	564	565	529
F1 Fetal data				
# Corpora lutea/doe	11.3 ± 2.2	12.0 ± 2.4	12.2 ± 2.2	12.6 ± 2.4
# implantations/doe	9.7 ± 2.2	9.0 ± 2.6	8.9 ± 2.4	9.5 ± 2.9
# Early resorp./doe	0.3 ± 0.5	0.3 ± 0.6	0.5 ± 0.9	2.7 ± 3.3*
# Late resorp./doe	0.5 ± 0.9	0.1 ± 0.4	0.1 ± 0.3	0.5 ± 1.1
# living pups/doe	8.9 ± 2.4	8.5 ± 2.4	8.4 ± 2.9	6.2 ± 3.2*
# dead pups/doe	0.0 ± 0.00	0.1 ± 0.3	0.0 ± 0.00	0.1 ± 0.3
Mean litter size	8.9 ± 2.4	8.6 ± 2.5	8.4 ± 2.9	6.3 ± 3.2*
Mean Fetal Weights	34.5 ± 4.3	37.6 ± 4.5	36.7 ± 5.3	38.1 ± 4.3
% Preimplantation loss[b]	14.7	25.0*	26.9**	24.9*
% Postimplantation loss[c]	8.3	5.2	6.4	34.5*

[a] -Body weight gains days 2 through 28 of pregnancy.
[b] -[(# corpora lutea - implant sites)/corpora lutea] x 100
[c] -[(#implant sites - viable fetuses)/implant sites] x 100
* -Significantly greater than control values ($p < 0.5$)
** -Significantly greater than control values ($p < 0.01$)

Briefly, the data in Table 10 show that treatment with r-Hirudin was associated with a slight, but non dose-dependent increase in preimplantation loss (average loss of approximately 3.0 to 3.1/dam in all treated groups versus 1.67/dam in control groups) out of average of 11.3 corpora lutea/dam in control groups and 12 to 12.6 corpora lutea in treated groups. Table 10 also shows that the 30 mg/kg dose was embryotoxic with increased number of resorptions (33.8% versus 8.3% in control females; 41 of 48 total resorptions were early) and increased post implantation loss (34.5% versus 8.3% in controls). As a result the mean litter size was also reduced (29%) in the 30 mg/kg dose groups.

F1 offspring from dames treated with r-Hirudin showed external anomalies and malformations which were limited to exencephaly in 1 of 129 fetuses examined at the 1 mg/kg dose and umbilical hernia in 1 of 94 fetuses evaluated at the 30 mg/kg dose. The low and non dose-dependent incidence of the said malformations suggest that neither was treatment-related. Tables 11 and 12, below present tabulated summaries of the observed soft tissue anomalies/malformations and the observed skeletal variations, anomalies and malformations, respectively.

Table 11. Incidence of Skeletal Variations and Anomalies in F_1 Fetuses from a Segment II Reproductive Toxicity Testing in ~~Rats~~ Rabbits

Treatment	Vehicle	r-Hirudin		
Dose (mg/kg)	0	1	10	30
Number of Litters Evaluated	15	15	14	15
Number of Fetuses Evaluated	133	128	117	93
	No. of Fetuses/No. of Litters Affected			
Soft Tissue Anomalies				
Gaseous Dilation of Stomach.....	7/3	1/1	10/2	2/1
Reddish Liquid in Stomach.....	0/0	1/1	0/0	0/0
Dilated Renal Pelvis.....	0/0	1/1	0/0	0/0
Soft Tissue Malformations:				
Brain				
Ventricular Cerebral Dilation.....	1/1	0/0	0/0	2/2
Kidney; Absence.....	0/0	1/1	0/0	0/0

Note: Anomalies refer to slight non-permanent and not obviously detrimental changes which occur in less than 10% of the historical control animals.